Adenosine monophosphate-activated protein kinase activator inhibits activation of fibroblast-like synoviocytes but promotes hyaluronan and proteoglycan link protein 1 secretion

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Abstract

Objectives: To determine whether any correlation exists between disease activity and AMPK levels in rheumatoid arthritis (RA) patients and investigate the effects of AMPK activator treatment on RA fibroblast-like synoviocytes (RA-FLS).

Methods: Serum AMPK-α1, p-AMPK-α1, TNF-α and IL-17 levels between osteoarthritis (OA) and RA patients having different disease activities were compared by ELISA. Differentially expressed genes (DEGs) between RA and OA synovium from NCBI GEO Profiles (accession numbers: GSE1202112, GSE55235, GSE5545713) were identified and the genes intersecting in all the three datasets were selected for enrichment analysis. Immunohistochemical staining was done with synovium obtained from OA and RA patients for p-AMPK-α1. AMPK gene expression in synovium was semi-quantified by RT-qPCR. RNA sequencing of FLS was performed and DEGs were selected for KEGG enrichment analysis. AMPK activator, metformin, treated RA-FLS were tested for proliferation and migration by MTT and scratch test, respectively. Expression of IL-6, AMPK-α1, PKA-α, RAPTOR, mTOR, HAPLN1, RUNX1 and RUNX2 genes were determined by qPCR. Phosphorylated AMPK-α1 and HAPLN1 levels were determined by an automated electrophoresis-western blot analysis method.

Results: In RA sera, a positive correlation between p-AMPK-α1 levels and DAS28 ($r = 0.270$, 95%CI: 0.142-0.492, $p < 0.0001$) as well as CRP levels ($r = 0.259$, 95%CI: 0.009-0.478, $p < 0.05$) was found. Similarly, a positive correlation was observed between AMPK-α1 and TNF-α levels ($r = 0.460$, 95% CI: 0.241-0.640, $p = 0.0002$). DEGs between OA and RA synovium from NCBI GEO profiles and our RNA sequencing data suggested activation of metabolic pathways specific to RA-FLS. AMPK-α1 was highly expressed in the synovium of RA but not OA patients. Metformin at higher concentrations inhibited RA-FLS proliferation in a dose dependant manner, however, at lower concentrations it has an opposite effect. On the other hand, AMPK inhibitor, dorsmophin, promoted the proliferation of RA-FLS significantly. Interestingly, both metformin and dorsmophin substantially inhibited the migration of RA-FLS. In FLS, relative expression level of IL-6 mRNA was significantly decreased after metformin treatment, while the expression of AMPK-α1, PKA-α and HAPLN1 genes were significantly increased. Western blot analysis confirmed increased expression of p-AMPK-α1 and HAPLN1 genes in the metformin treated FLS.

Conclusions: Inflammatory stress in RA synovium leads to an increase in AMPK levels, possibly as a protective mechanism. AMPK activator but not metformin *per se* could be a potential therapeutic for RA by promoting HAPLN1 secretion to protect the joints.

Introduction

Growing evidences show the importance of metabolic variations in several autoimmune diseases. For example, inflammation associated macrophages and T-helper 17 cells display a shift towards enhanced glucose uptake, glycolysis and an increased activity of the pentose phosphate pathway. In contrast, anti-inflammatory cells like M2 macrophages, regulatory T cells and quiescent memory T cells exhibit lower

glycolytic rates and higher levels of oxidative metabolism (1). The role of fibroblast-like synoviocytes (FLS) in the pathogenesis of rheumatoid arthritis (RA) is increasingly appreciated and recognized as key effector cells (2). The synovium in RA transforms from a quiescent state with relatively acellular structure to a hyperplastic, invasive tissue infiltrated with many inflammatory cells. While the activated FLS are hyper-proliferative to form pannus and acquire resistance to apoptosis, their migration and mobility capacities are also increased. These properties contribute to the invasion potential of FLS, which by producing inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interlukin-17 (IL-17), chemokines, and matrix-degrading molecules (3) contribute to the destruction of joint cartilage. Recently, choline metabolism was reported to be activated in RA-FLS. Hence, the metabolic variations in RA-FLS could potentially lead to identification of novel signaling pathways and therapeutic agents (4).

Adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved metabolic fuel gauge in eukaryotes that senses changes in the intracellular AMP/ATP ratio in response to energy deprivation by regulating mitochondrial biogenesis (5). Studies have reported that AMPK plays an important role in several inflammatory pathways. For example, AMPK controls the transcriptional regulation of autophagy and lysosomal genes (6); promotes autophagy through phosphorylation of Unc-51 like autophagy activating serine/threonine protein kinase Ulk1 (7); attenuates CD40-mediated inflammatory activities; toll-like receptor (TLR)-induced inflammatory functions and decreases the capacity of antigen presenting cells (8). Some anti-inflammatory agents may function by activating AMPK, a state akin to pseudo-starvation, which alter the metabolism and may participate in the signal-directed programs either by promoting or inhibiting inflammation (8). In this study, we analyzed the correlation between AMPK levels and disease activity in RA patients. Although metformin, as an AMPK activator, was tested in experimental arthritis and reported to down regulate Th-17 cells (10), its effect on FLS is not clear.

Materials And Methods

Patients' samples

Sixty-one RA and twenty OA patients were enrolled for this study (for patients' details see supplementary file 1) with written informed consent obtained from all the patients. Institutional ethical guidelines were followed as per Helsinki declaration. Patients' sera were collected and AMPK-α1, p-AMPK-α1, TNF-α and IL-17 levels were determined using ELISA kits by following manufacturer's instructions (R&D Systems, USA). Synovium samples from 20 RA and 17 OA patients were collected during arthroscopic surgery done for therapeutic purposes. Streptavidin-biotin based immunoperoxidase staining for p-AMPK-α1 was performed using formalin fixed, paraffin embedded specimens (For detailed methods, see supplementary file 1). Image J was used for analysis.

Analysis of differentially expressed genes between RA and OA synovium

We analyzed the data of synovial tissues from RA and OA patients obtained from NCBI GEO Profiles (accession numbers: GSE12021(11), GSE55235 and GSE55457(12)). The common differentially expressed genes (DEGs) between RA and OA patients intersecting in all the three datasets were selected

for enrichment analysis by submitting to KOBAS 3.0 web tool (http://kobas.cbi.pku.edu.cn/) for KEGG pathway enrichment analysis. Results with corrected p values < 0.05 were selected to plot the bubble diagram.

Quantitative real-time polymerase chain reaction (qPCR)

Synovium specimens from 10 RA and 9 OA patients obtained through knee arthroscopy (for patients' details, see supplementary file 1) were soaked in TRIzol® Reagent (Thermo Scientific, USA) after removing the adipose tissues under aseptic conditions. All the samples were stored in -20℃ freezer until used. Expressions of AMPK-α1, AMPK-α2, AMPK-γ1 and AMPK-γ3 genes were analyzed by qPCR (for methods, see supplementary file 1). To evaluate the effects of metformin on FLS, relative mRNA expression levels of IL-6, AMPK-α1, PKA-α, RAPTOR, mTOR, HAPLN1, RUNX1 and RUNX2 genes were measured by qPCR (Primers list is given in supplementary file1).

Isolation and culture of RA- FLS

FLS were derived from synovial tissue specimens harvested from patients by needle arthroscopy. FLS were isolated by enzyme digestion and subsequently cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS, Invitrogen) containing antibiotics (penicillin and streptomycin) at 37°C with 5% CO_2. Cells cultured between passages 4 and 9 were used for this study. Cells were frozen with cell freezing medium and stored in -80℃ freezer until used.

High-throughput mRNA sequencing

High-throughput RNA sequencing was performed with FLS from RA and OA patients (n = 3/group) (for patients' information, see supplementary file 1). Gene expression differences between RA and OA-FLS were investigated using KEGG pathway enrichment analysis.

Methyl thiazolyl terazolium (MTT) assay

MTT assay was used to determine the effects of metformin and dorsomorphin on FLS viability at different concentrations (For detailed method, see supplementary file 1).

Scratch test

Scratch test was performed to evaluate the effects of metformin and dorsomorphin on FLS migration viability (for detailed method, see supplementary file 1).

Automated electrophoresis western blot analysis

FLS treated with metformin (5 mM) or saline for 36 h were examined for relative changes in p-AMPK-α1 and hyaluronan and proteoglycan link protein 1 (HAPLN1) levels by automated electrophoresis followed by western blot analysis (for detailed methods, see supplementary file 1).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. All the data were given as mean ± SD. Differences between two groups were evaluated for statistical significance using Student's t-test. One-way ANOVA with Tukey's multiple comparisons test was used to evaluate the differences among three or more groups. Correlations were evaluated using Liner regression and correlation test. $p < 0.05$ was considered as statistically significant.

Results

Serum p-AMPK-α1 levels positively correlated with disease activity in RA

To investigate the difference in AMPK levels between RA and OA patients and to detect the presence of correlation between AMPK levels and RA disease activity, we determined AMPK-α1 and p-AMPK-α1 levels using 20 OA and 61 RA patients having different disease activities. No significant differences exist in AMPK-α1 levels between OA and RA patients (Figure 1A). However, p-AMPK-α1 levels were higher in OA compared to RA patients, who had lower disease activity ($p < 0.01$) (figure 1B). This significance level was increased when the values for p-AMPK-α1 levels were log transformed ($p < 0.0001$). Interestingly, after log transformation, RA patients having higher disease activity was found to have significantly higher levels of p-AMPK-α1 compared to patients having low disease activity ($p < 0.01$) (Figure 1C). In addition, p-AMPK-α1 levels were positively correlating with DAS28 scores ($r = 0.270$, 95%CI: 0.142 - 0.492, $p < 0.0001$) and CRP levels ($r = 0.259$, 95%CI: 0.009 - 0.478, $p < 0.05$) (Figure 1D and E). However, such a correlation did not exist with ESR levels (Figure 1F).

IL-17 and TNF-α levels were reported to have correlation with RA activity and thus were currently selected as therapy targets (13, 14). We analyzed the presence of possible correlation between AMPK levels with IL-17 and TNF-α in the serum samples. The results demonstrated an increased expression of both the inflammatory cytokines in RA than OA patients, and AMPK-α1 levels were moderately correlated with TNF-α levels ($r = 0.46$, 95% CI: 0.241-0.640, $p = 0.0002$) (Figure 1G, H & I). However, no statistical correlation exists between log transformed p-AMPK-α1 and IL-17 or TNF-α levels (supplementary file 2).

Although AMPK is a key regulator of glucose metabolism (15), and RA and other autoimmune diseases appear to be associated with an increased risk of diabetes mellitus (16), no differences in glucose levels between groups were observed in our study (supplementary file 2).

AMPK levels were more significantly present in RA synovial samples

Since AMPK has an anti-inflammatory effect in many inflammation related diseases (17, 18), and our data showed a mild positive correlation with disease activity, we next evaluated the data available in NCBI GEO Profiles [accession numbers: GSE12021(11), GSE55235, and GSE55457(12)] for AMPK levels from RA and OA patients' synovial tissue samples. In total, 119 DEGs intersected in all the three datasets (supplementary file 3), which demonstrated the high consensus existing between results from different

experiments. Although AMPK was not directly detected in the analysis, one of the most obviously changed pathway was the metabolic pathway consisting of 9 genes (Figure 2A). Since AMPK has a controlling function in metabolism (6, 19), we focused on its expression at both protein and mRNA levels in the synovium samples from OA and RA patients. The immunohistochemical staining revealed higher level of p-AMPK-α1 expression in RA than OA synovium (Figure 2B). Similarly, relative expression levels of AMPK-α1, AMPK-α2 and AMPK-γ3 genes were higher in synovium of RA than OA patients (Figure 2C).

Metabolism variations in FLS

Phosophorylated AMPK-α1 expressed in the proliferating FLS identified using IHC staining was confirmed by immunofluorescence studies (see supplementary file 2). FLS are the most common cell types present at the pannus-cartilage junction, which contribute to joint destruction through their production of cytokines, chemokines and matrix-degrading molecules as well as by migrating and invading the joint cartilage (20), and thus regarded as key effector cells(21). Hence, we analyzed the differences in the expression of genes between RA and OA FLS by high-throughput RNA sequencing method. The results demonstrated that among the 111 DEGs identified, expression of 95 genes were up-regulated and 16 genes were down regulated in RA than OA FLS (Figure 3A and to see all the DEGs present in the individual samples refer supplementary file 4). KEGG pathway enrichment analysis suggested involvement of metabolic and glycerolipid metabolism pathways with 9 genes closely related to AMPK regulation (Figure 3A, B). Among them, diacylglycerol kinase gamma (DGKG) and prostaglandin D2 synthase (PTGDS) expressions were down regulated, while the expression of other 7 genes, lipase F (LIPG), heparanase (HPSE), glycerol-3-phosphate acyltransferase 2 (GPAT2), phospholipase A2 group VII (PLA2G7), choline dehydrogenase (CHDH), ST6 b-galactoside a-2,6-sialyltransferase 2 (ST6GAL2) and ST8 a-N-acetyl-neuraminide a-2,-sialyltransferase 5 (ST8SIA5) were up regulated in RA compared to OA FLS. Interestingly, hyaluronan and proteoglycan link protein 1 (HAPLN1) expression was highly up-regulated in RA-FLS (Figure 3A)

Metformin affects FLS proliferation

Based on our above results and prior knowledge on anti-inflammatory functions of AMPK (7, 8), we deduced that up-regulation of AMPK-α1 expression in RA synovium might be due to an inflammation stress. So, we used metformin (22) and dorsmorphin (23) as AMPK activator and inhibitor, respectively to evaluate their effects on FLS proliferation.

FLS were cultured with different concentrations of metformin or PBS in 6-well plates and observed between 0-72 hours under inverted microscope for their viability. MTT assay was performed to confirm the cellular viability of FLS after metformin treatment. Both the results demonstrated inhibition of FLS proliferation by metformin at 5 and 10 mM concentrations. However, at a low concentration (1 mM) metformin promoted FLS proliferation (Figure 4A, B), which was further confirmed by reducing the concentration further. Importantly, metformin inhibited FLS proliferation even at 2 mM concentration (Figure 4C), demonstrating dose-dependent effect of metformin on FLS proliferation. In contrast, dorsmorphin promoted FLS proliferation significantly at very low (5 and 10 μM) concentrations.

Metformin inhibited FLS migration

Based on the concentration gradient of metformin for its inhibitory effects on FLS proliferation, we selected metformin at 5 mM as well as dorsmorphin at 5 μM to study their effect on FLS migration ability by testing wound repair rate in a scratch test experiment. Interestingly, both metformin and dorsmorphin inhibited FLS migration significantly (Figure 5). Earlier, dorsmorphin was reported to inhibit the migration of certain cancer cells and this phenomenon was explained by AMPK-independent mechanisms (23).

Metformin increased AMPK-α1 and HAPLN1 expression

Results from semi-quantification of mRNA levels in FLS after metformin treatment by RT-qPCR showed a significant decrease in IL-6 gene expression, while the expressions of AMPK-α1, PKA-α and HAPLN1 genes were significantly increased (Figure 6A). Automated electrophoresis western blot analysis confirmed the up regulation of p-AMPK-α1 and HAPLN1 at the protein level (Figure 6H).

The role of IL-6 in the pathogenesis of joint and systemic inflammation in RA has been clearly demonstrated (24), and IL-6 inhibitor has been used for the treatment of RA with favorable outcomes (25). Our results confirmed AMPK-dependent effects of metformin on IL-6 gene expression (26, 27) as we noticed a significant negative correlation ($r = -0.422$, 95%CI: -0.672 to -0.0865, $p = 0.016$) between AMPK-α1 and IL-6 gene expressions (Figure 6B). PKA-α is a regulatory subunit of the cAMP-dependent protein kinases involved in cAMP mediated signaling events in the cells and a mutual promotion effect between AMPK and PKA-α had been reported earlier (28, 29). In this study, we found a significant increase in PKA-α gene expression in FLS after metformin treatment. AMPK also phosphorylates the mammalian target of rapamycin complex 1 (mTORC1) subunit, regulatory associated protein of mTOR (RAPTOR), which is essential for AMPK function as a metabolic checkpoint (30). Although RAPTOR and mTOR did not have any significant changes after metformin treatment, a negative correlation between RAPTOR and AMPK-α1 expression was detected ($r = -0.470$, 95%CI: -0.682 to -0.185, $p = 0.002$) (Figure 6C) confirming an earlier report (31). This pathway was reported to regulate cell growth in response to nutrient and insulin levels.

Interestingly, after treating FLS with metformin, an up-regulation of HAPLN1 expression was observed, which was significantly positive correlated with AMPK-α1 gene expression ($r = 0.560$, 95%CI: 0.308 to 0.738, $p < 0.0001$, Figure 6D) as well as at protein level ($r = 0.785$, 95%CI: 0.3869 to 1.238, $p = 0.0015$, Figure 6I). HAPLN1 was reported as one of the distinctive genes expressed in RA FLS correlating with the disease activity (32). However, effects of metformin on AMPK-α1 expression and subsequent modulation of HAPLN1 has not been reported earlier. HAPLN1 interacts with the globular domains of hyaluronic acid and proteoglycans to form stable ternary complexes in various extracellular matrices. Its main biological function is to maintain the stable aggregation of hyaluronic acid and proteoglycan monomers in the extracellular cartilage matrix (33), and to stabilize the binding interactions between hyaluronic acid and chondroitin sulfate, which contribute to the compression resistance of joints (34). Perinatal mice containing targeted mutations in the HAPLN1 gene developed lethal cartilage dysplasia (35) suggesting the essential role of HAPLN1 as a regulator of cartilage homeostasis and formation.

In granulosa cells, HAPLN1 was proposed to be promoted through PKA-RUNX1/RUNX2 pathway (36). Although RUNX1 and RUNX2 expressions in metformin treated FLS were not significantly changed, RUNX1 expression was negatively correlating with AMPK-α1 expression (r = -0.339, 95%CI: -0.604 to -0.007, p < 0.046), while having a positive correlation with HAPLN1 (r = 0.547, 95%CI: 0.291 to 0.729, p < 0.0001, Figure 6E and G) expression. Conversely, HAPLN1 expression did not show any significant correlation with RUNX2, though AMPK-α1 and RUNX2 expressions were positively correlated (r = 0.471, 95%CI: 0.103 to 0.656, p = 0.011, Figure 6F). However, our current study cannot explain this contradictory observation because of the prevailing extremely complicated molecular interactions *in vivo*. RUNX1 controls anergy and suppressive functions of regulatory T-cells by associating with FOXP3. It activates the expression of IL-2 and IFN-γ and down-regulates the expression of TNF receptor superfamily member 18 (TNFRSF18), IL-2 receptor subunit alpha (IL-2RA) and cytotoxic T-lymphocyte associated protein 4 (CTLA4) in conventional T-cells(37), while positively regulating the expression of RORC in T-helper 17 cells (38). On the other hand, RUNX2 is essential for the maturation of osteoblasts and has an important role in the intramembranous and endochondral ossification processes (39, 40). Based on our knowledge, we summarized possible mode of action of metformin on FLS in figure 5 of online supplementary file 2.

Discussion

AMPK is one of the most important energy sensors identified so far that can regulate the cellular metabolism. Similarly, AMPK inhibits a broad range of inflammatory reactions through multiple biological pathways by regulating immune cell metabolism and functions (41, 42). In our current study, no obvious differences in the serum levels of AMPK-α1 and p-AMPK-α1 between OA and RA patients were observed. However, AMPK-α1 and log (p-AMPK-α1) levels were moderately positive correlated with the disease activity in RA patients. Moreover, p-AMPK-α1 expression was higher in RA than OA synovium. AMPK is very sensitive to various stimulations, hence differences in its level may not be reflected well in the serum samples. On the other hand, in the synovium of joints where all the inflammatory reactions occur (43), an up-regulation of AMPK is more clearly distinct, though cause and effect relationship between AMPK and RA is not yet clear. Possibly, inflammation induced stress in the synovium could lead to an increase in AMPK secretion level.

Though metformin as an AMPK activator had inhibitory potential at higher concentrations, it promoted FLS proliferation at lower concentrations in our hands, which is in disagreement with the study of Chen et al (44). They have reported inhibitory capacity of metformin even at very low concentrations (5-60 μM) on FLS proliferation. Since the dose differences used in both the studies were very high, difference in metformin preparations obtained from different manufacturers may not be the major cause. It is plausible that differences in the source of FLS, patient characteristics, genetic variations and treatment conditions could have contributed to these contradictory observations. Most publications reported inhibitory effect of metformin mainly using cancer cells (45, 46). An analogy to our observation is the use of methotrexate and radiation, which can be used to treat cancer while being carcinogenic. In fact, pharmacological concentrations of metformin at low or high doses affect cells by different mechanisms (47). At a higher concentration (5 mM), it was proposed to inhibit the respiratory chain complex 1 in intact

hepatocytes causing an increase in the AMP/ATP ratio (48). However, more studies are needed to clarify this issue.

Our results are in accordance with metformin effects on the activation of AMPK and in attenuating inflammation (49). We have observed down regulation of IL-6 gene expression, while expression of PKA-α, which exerts a negative effect on AMPK activation (50) was found to be up regulated by metformin treatment. Interestingly, we have identified up-regulation of HAPLN1 expression, which showed a positive correlation with AMPK-α1. In mesenchymoma tissues, HAPLN1 is significantly elevated at both RNA and protein levels (51). HAPLN1 reappears in aggressive hepatocytes that express cytoplasmic β-catenin and stem cell markers, and it is associated with poor disease outcomes (52). HAPLN1 is also a susceptibility gene for lung cancer (53).

In RA, Tomohiko et al (54) studied the correlation between HAPLN1 gene polymorphism and spinal degenerative changes in 622 postmenopausal Japanese women and showed that mutations in the specific HAPLN1 loci are related to spinal degeneration. William et al. have established a model of cartilage dystrophy in dogs to mimic the degenerative changes of intervertebral discs and nucleus pulposus, and found a higher level expression of HAPLN1 protein in the model group than the controls. Furthermore, genome-wide association analysis reported HAPLN1 as one of the important susceptibility genes for ankylosing spondylitis in the Han population (55). In RA-FLS, expression of HAPLN1 gene was positively correlating with C-reactive protein that reflects disease activity (56).

Our RNA sequencing results demonstrated an up-regulation of HAPLN1 expression in RA-FLS. This led us initially to speculate HAPLN1 as a pathogenic factor in RA-FLS, because it mimics the condition of cancer cells in proliferation and migration leading to cartilage destruction (21). However, anti-inflammatory effects of AMPK, and its positive correlation with HAPLN1, together with the critical role of HAPLN1 reported in cartilage homeostasis (36) suggest a need for more functional experiments with HAPLN1 in samples from RA patients.

Conclusion

In summary, we tested metformin as an AMPK activator and found its inhibitory role on RA-FLS proliferation and migration. Metformin also affected the expression of many inflammatory and metabolic molecules. Surprisingly we found that at lower concentrations metformin increased the proliferation of RA-FLS. Considering the dosage used in the *in vitro* studies, it is most unlikely that metformin will be used as an effective drug for the treatment of RA patients. However, it is clear that AMPK pathway is a valuable target for treatment and developing new drugs targeting AMPK activation might be more beneficial for protecting the joints in RA patients.

Abbreviations

AMPK: Adenosine monophosphate-activated protein kinase

CRP: C-reactive protein

DEG: different expression gene

Dor: dorsmorphin

ESR: erythrocyte sedimentation rate

HAPLN1: hyaluronan and proteoglycan link protein 1

H: high

HPF: high power field

IL: interleukin

L: low

Met: metformin

M: moderate

MTT: methyl thiazolyl terazolium

OA: osteoarthritis

OD: optical density

qPCR: quantitative real-time polymerase chain reaction

TNF: tumor necrosis factor

RA: rheumatoid arthritis

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Integrated Traditional Chinese and Western Medicine hospital, Southern Medical University, China (approval No. NFZXYEC-2017-002). Informed consent was obtained from all the participants in accordance with the declaration of Helsinki.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the first author or corresponding author on reasonable request.

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Figures

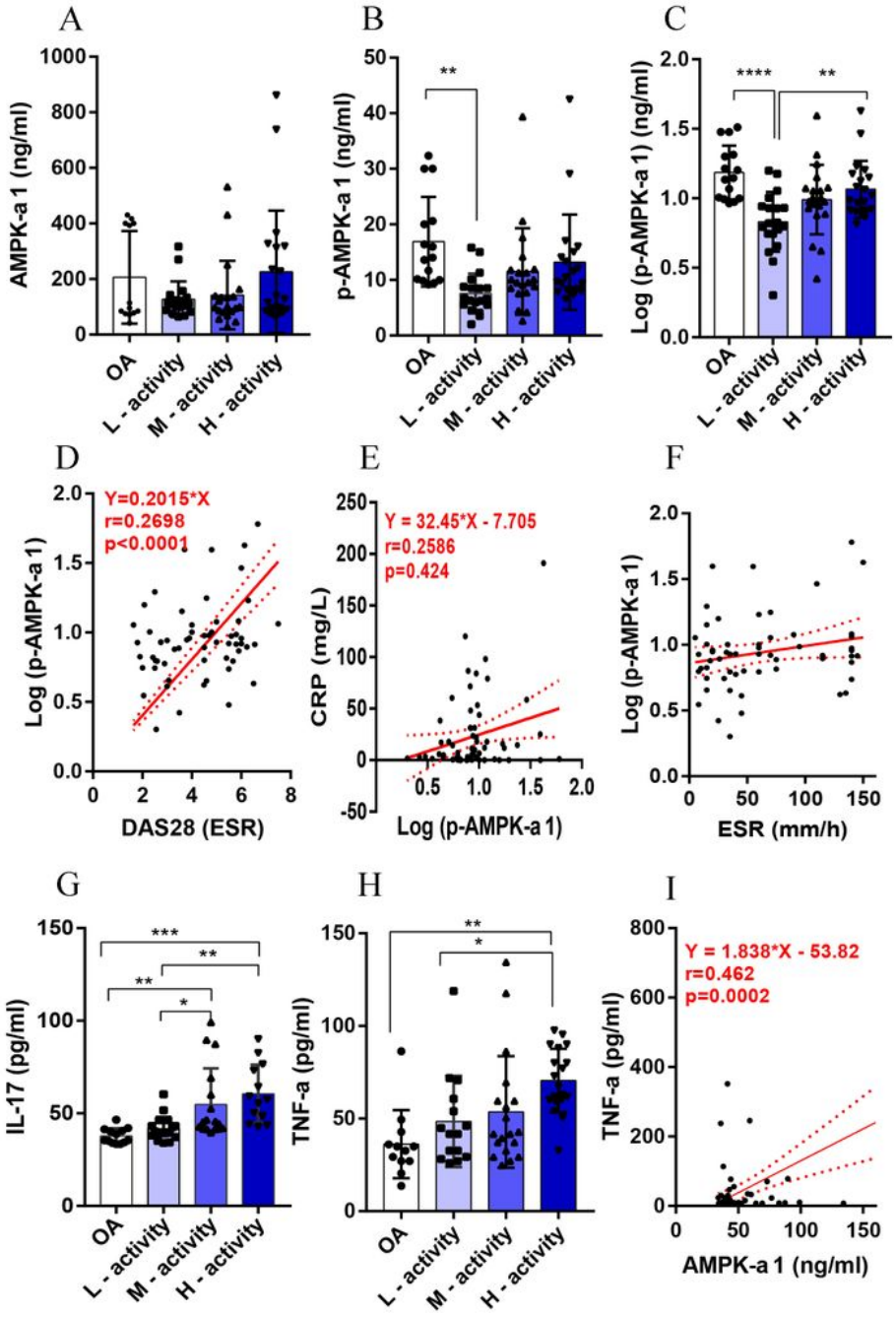

Figure 1

Expression of serum AMPK-α1 and p-AMPK-α1 levels in RA patients. AMPK-α1 levels between OA and RA patients having moderate to high disease activity was shown (A). Serum p-AMPK-α1 level was higher in OA compared to RA patients having lower disease activity (B). Log transformed p-AMPK-α1 values were significantly higher in OA than RA patients having lower disease activity. Patients with higher disease activity had significantly higher levels of log transformed p-AMPK-α1 levels compared to patients with

lower disease activity (C). Log p-AMPK-α1 levels significantly correlated with DAS28 and CRP levels (D & E). Correlation with ESR levels was not statistically significant (F). Increased expression of IL-17 and TNF-α was observed in RA than OA patients (G and H). Levels of AMPK-α1 moderately correlated with TNF-α levels (I). *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. AMPK, AMP-activated protein kinase; RA, rheumatoid arthritis; OA, osteoarthritis; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IL, interleukin; TNF, tumor necrosis factor; L, low; M, moderate; H, high.

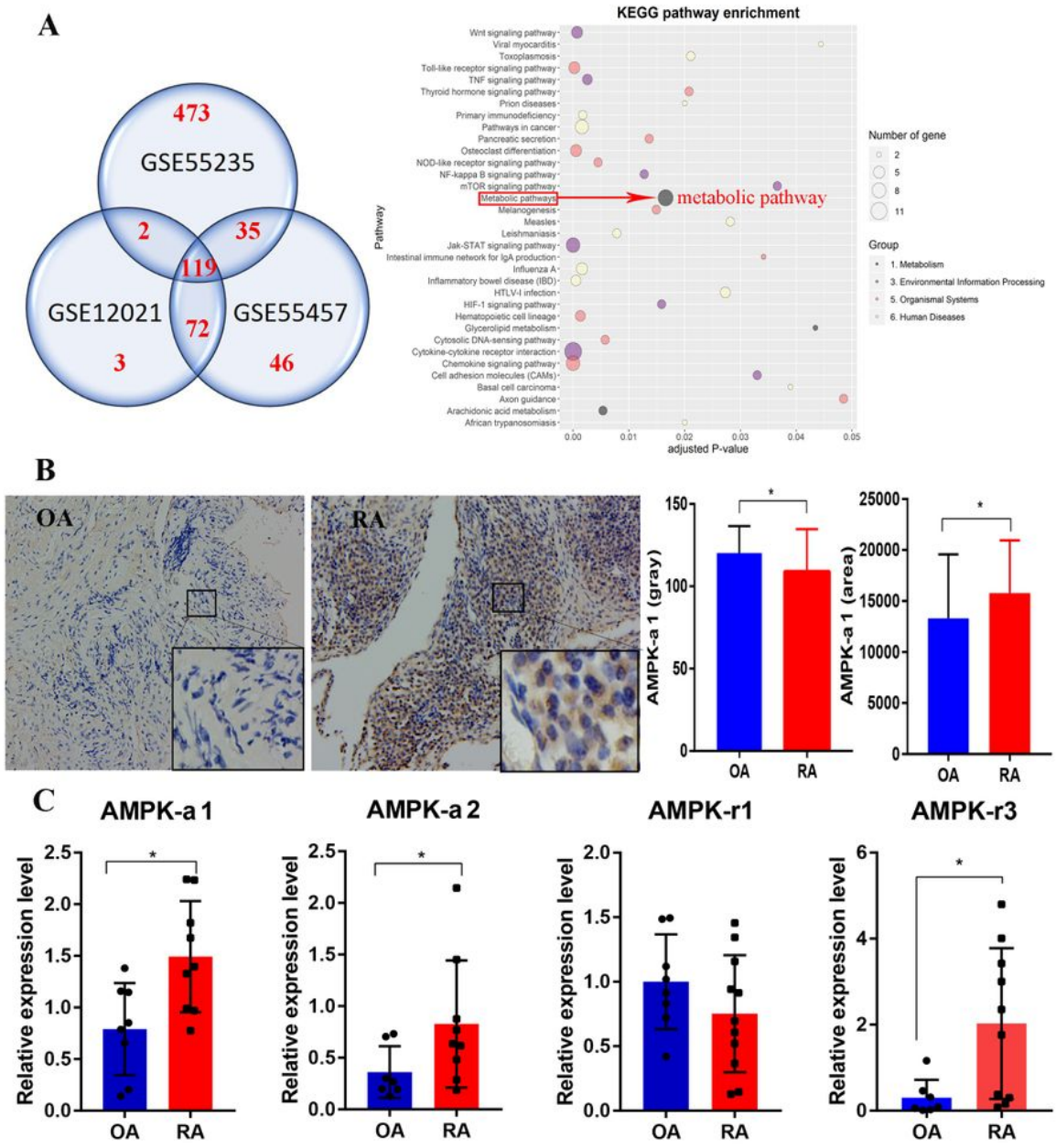

Figure 2

Changes in the metabolic pathway in RA compared to OA synovium. In total, 119 DEGs between RA and OA patients' synovium intersected in all the three datasets (NCBI GEO Profiles, accession numbers: GSE12021, GSE55235, GSE55457). Among them, changes in the metabolic pathway related 9 genes were more obvious (A). Staining for p-AMPK-α1 was more intense in RA than OA synovium (B). Relative expression levels of AMPK-α1, AMPK-α2 and AMPK-γ3 genes were higher in RA than OA synovium (C). *, $p < 0.05$.

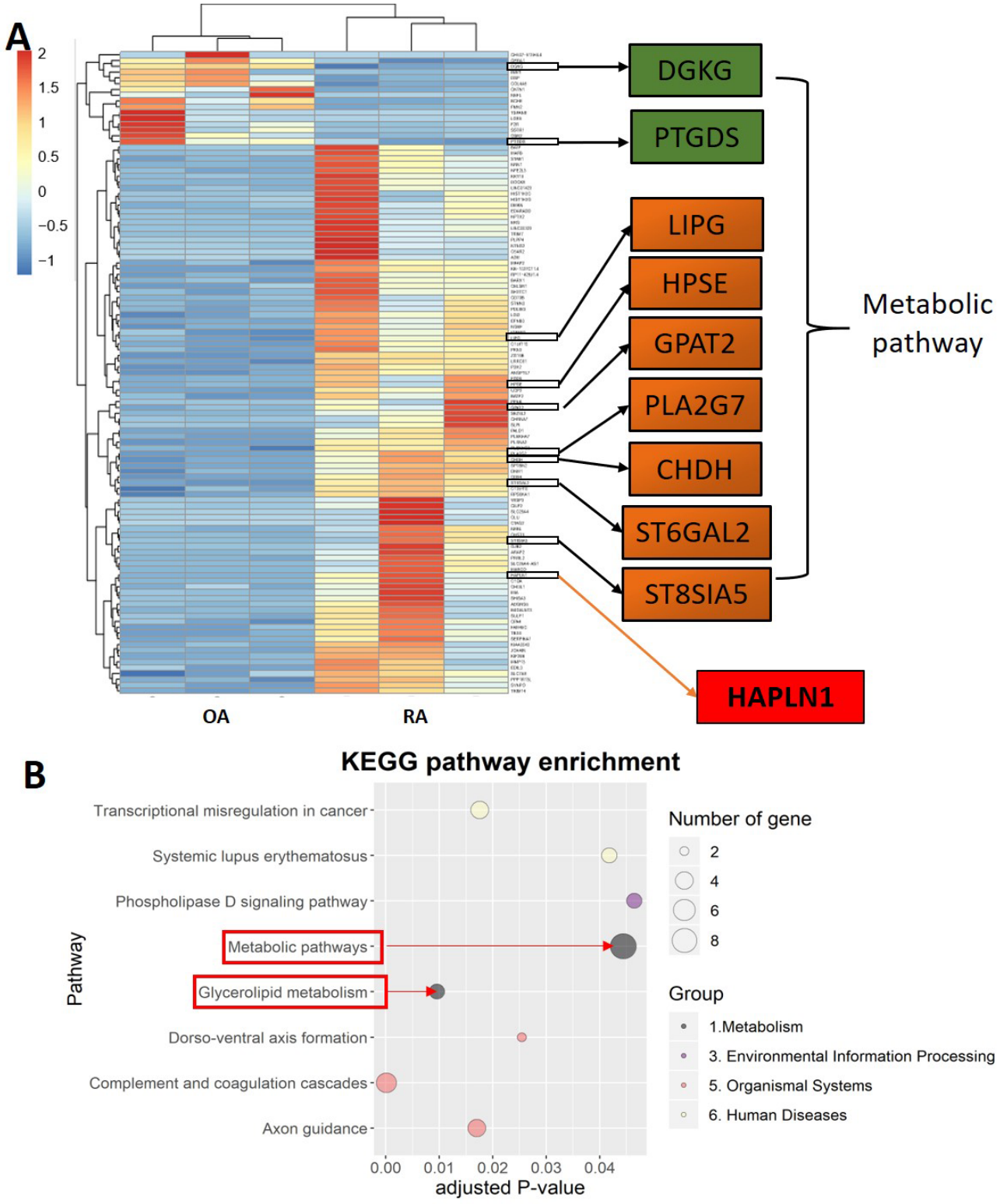

Figure 3

DEGs in FLS identified by mRNA high-throughput sequencing method. In total, 111 DEGs were identified in RA-FLS compared to OA samples. Expression of 95 genes was up-regulated compared to the down regulation in the expression of 16 genes. Of all the 9 DEGs related to metabolism, expression of DGKG and PTGDS genes were down regulated, while the expression of LIPG, HPSE, GPAT2, PLA2G7, CHDH, ST6GAL2 and ST8SIA5 genes were up-regulated in RA-FLS compared to OA samples. HAPLN1 expression was most obviously up-regulated in RA-FLS (A). KEGG pathway enrichment analysis demonstrated most obvious changes in the metabolic pathways (B).

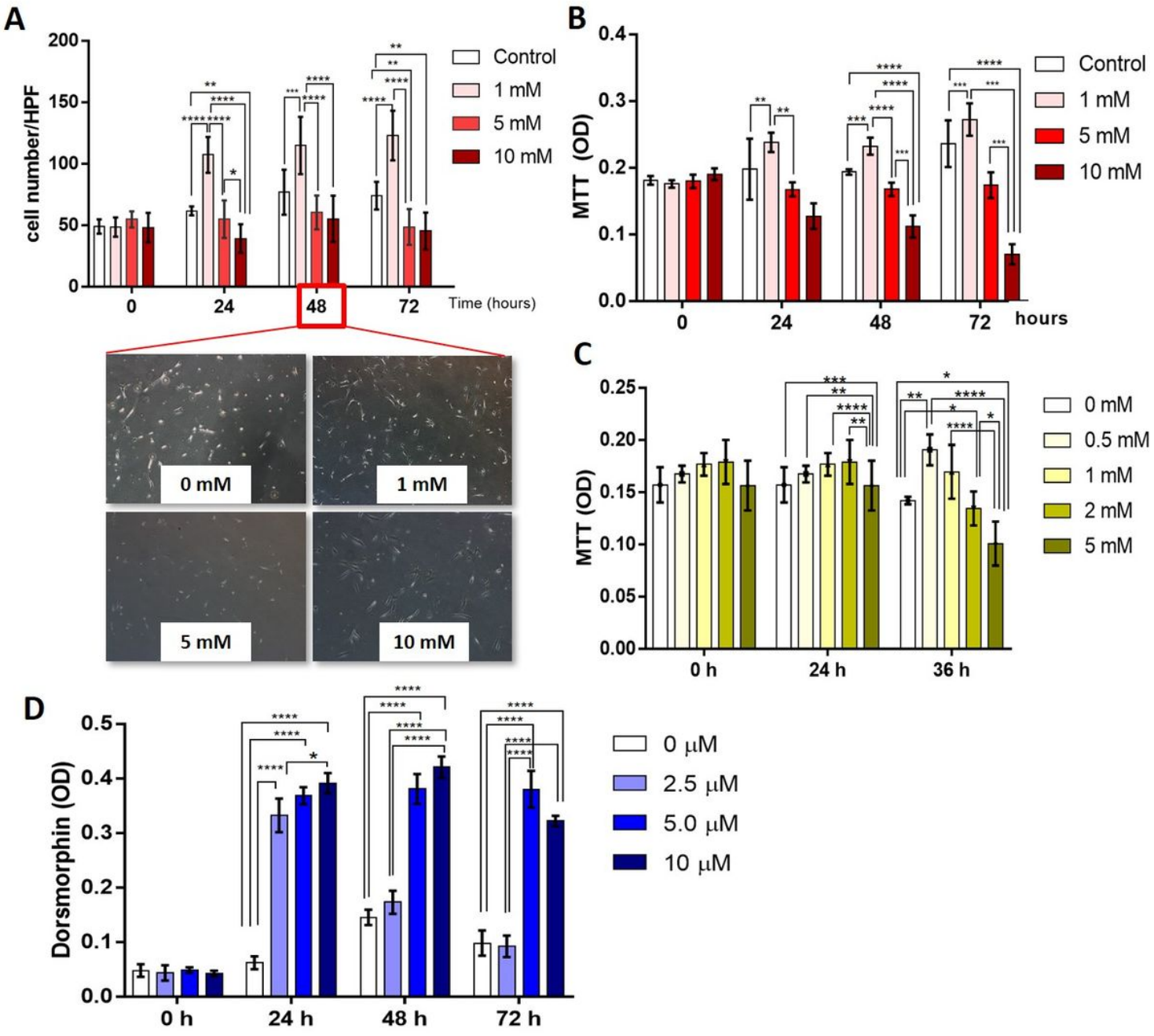

Figure 4

Effect of AMPK modulators on FLS proliferation. AMPK activator metformin inhibited FLS proliferation at 5 and 10 mM, whereas at 1 mM it has an opposite effect (A and B). This result was confirmed by CCK8

test (C). Dorsmorphin promoted FLS proliferation significantly at 5 and 10 μM (D). *, p < 0.05, **, p < 0.01; ***, p < 0.001; ****, p <0.0001; HPF, high power field; OD, optical density.

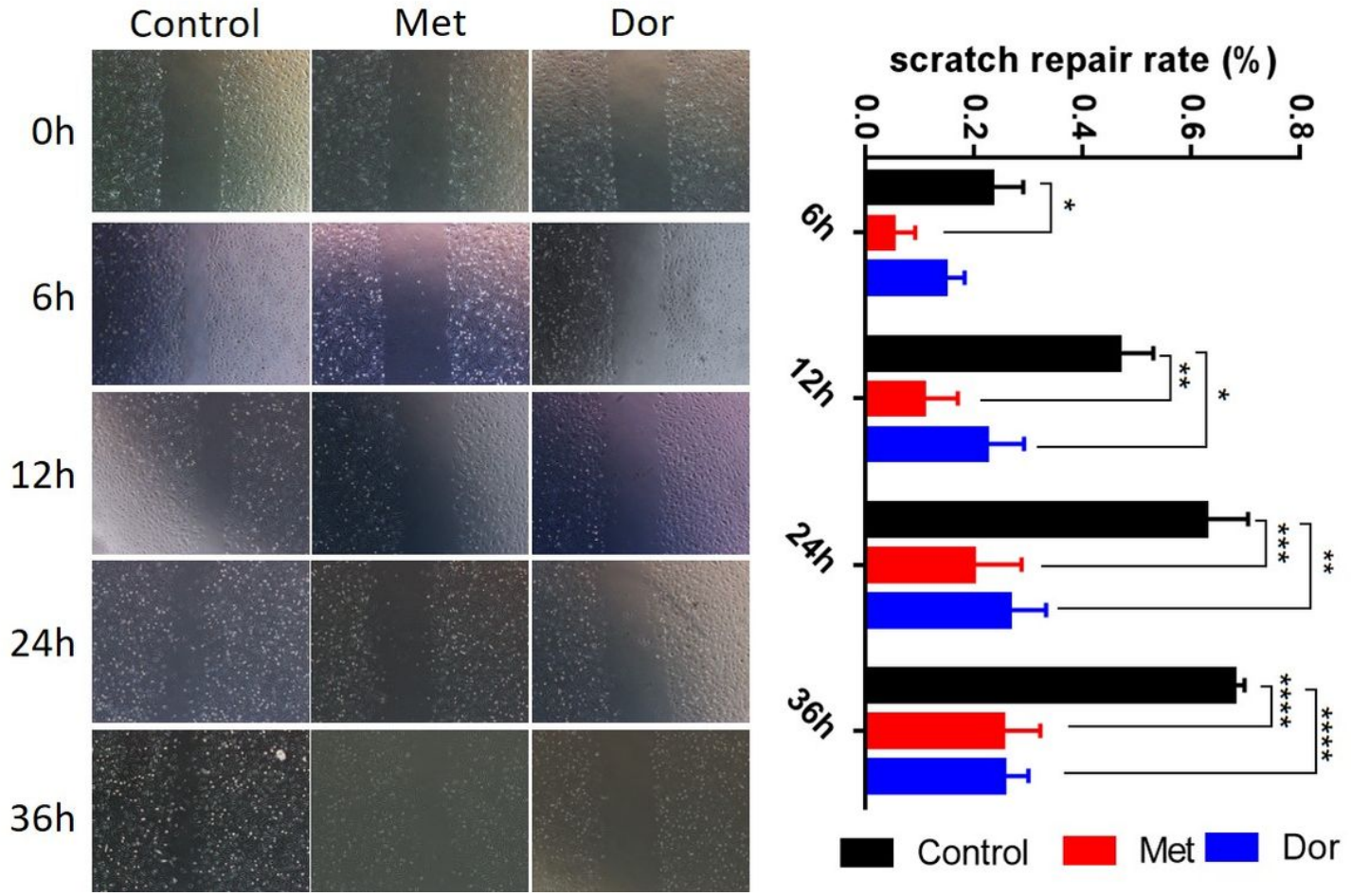

Figure 5

AMPK modulators inhibited FLS migration. Both AMPK activator, metformin and inhibitor, dorsmorphin showed inhibitory ability on FLS migration significantly. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; Met, metformin; Dor, dorsmorphin.

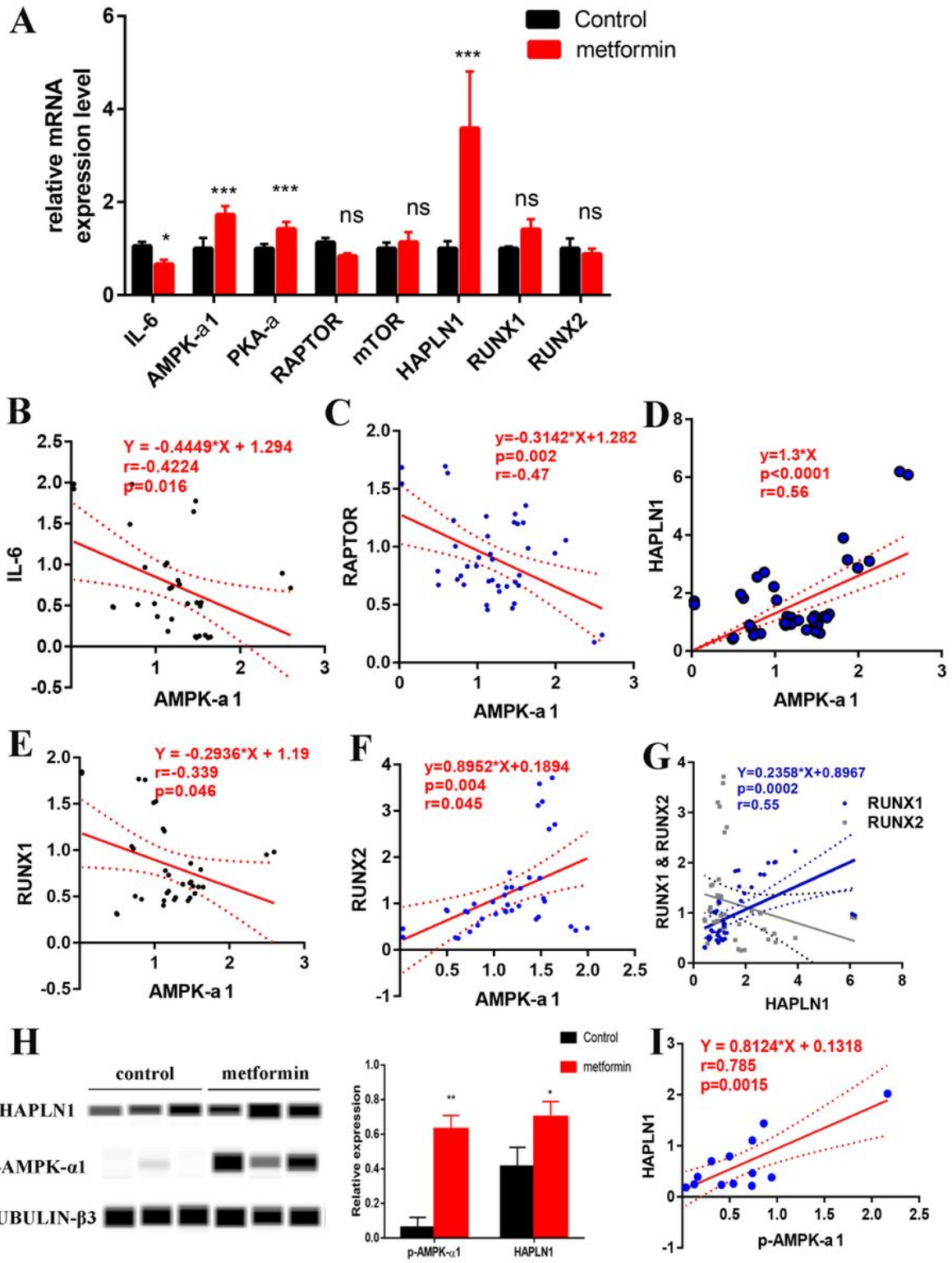

Figure 6

Metfomin treatment changed the relative mRNA expression levels in FLS. Compared to controls, expression of IL-6 gene was significantly decreased in metformin treated FLS, while the expressions of AMPK-α1, PKA-α and HAPLN1 genes were significantly increased (A). Significant negative correlations between AMPK-α1 and IL-6, AMPK-α1 and RAPTOR, AMPK-α1 and RUNX1 expressions were detected by liner regression analysis (B, C and E), while significant positive correlations between AMPK-α1 and

HAPLN1, RUNX1 and RUNX2 gene expressions were identified (D, F and G). Automated electrophoresis western blot analysis demonstrated an increased expression of p-AMPK-α1 and HAPLN1 in metformin treated FLS compared to the controls (H). A significant positive correlation between p-AMPK-α1 and HAPLN1 levels were detected by linear regression analysis (I).*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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